Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1 (G207)

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ABSTRACT **G207 is a multi-mutated, replication-competent type-1 herpes simplex virus designed to target, infect, and lyse neurological tumors. This study examines the feasibility of using G207 in the treatment of human colorectal cancer and defines the biological determinants of its antitumor efficacy. This virus was tested on five human colorectal cancer cell lines *in vitro* to determine efficacy of infection and tumor cell kill. These results were correlated to measures of tumor cell proliferation. *In vivo* testing was performed through direct injections of G207 into xenografts of human colorectal cancer tumors grown in flanks of athymic rats. To evaluate an alternate method of administration, hepatic portal vein infusion of G207 was performed in a syngeneic model of liver metastases in Buffalo rats. Among the five cell lines tested, infection rates ranged between 10% and 90%, which correlated directly with S-phase fraction (8.6%–36.6%) and was proportional to response to G207 therapy *in vitro* (1%–93%). Direct injection of G207 into nude rat flank tumors suppressed tumor growth significantly vs. control (0.58 ± 0.60 cm^3 vs. 9.16 ± 3.70 cm^3, $P<0.0001$). *In vivo* tumor suppression correlated with *in vitro* effect. In the syngeneic liver tumor model, portal infusion resulted in significant reduction in number of liver nodules (13 ± 10 nodules in G207-treated livers vs. 80 ± 30 nodules in control livers, $P<0.05$). G207 infects and kills human colorectal cancer cells efficiently. *In vitro* cytotoxicity assay and tumor S-phase fraction can be used to predict response to treatment *in vivo*. This antineoplastic agent can be delivered effectively by both direct tumor injection and regional vascular infusion. G207 should be investigated further as therapy for colorectal cancer and liver metastases.—Kooby, D. A., Carew, J. F., Halterman, M. W., Mack, J. E., Bertino, J. R., Blumgart, L. H., Federoff, H. J., Fong, Y. Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1 (G207). *FASEB J.* 13, 1325–1334 (1999)**

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COLORECTAL CANCER IS the third most common malignancy and the second leading cause of cancer deaths in the United States (1). Half of those affected with colorectal cancer will die of advanced or recurrent disease, despite aggressive treatment (2). The liver is the most frequent site of distant metastatic spread from malignancies of the colon and rectum (3, 4). Patients with untreated liver metastases have a median survival of 5 to 10 months (5, 6). Although many forms of therapy have been evaluated, only surgical resection of the affected liver offers the possibility of cure (7). Two-thirds of those who undergo successful resection, however, experience recurrence, presumably from microscopic residual disease (8). For patients with unresectable liver metastases, palliative treatment, in the form of chemotherapy, yields clinical response in only one-third of cases (9). Thus, active investigation seeks novel therapies that may improve outcome in both resectable and unresectable cases of this common disease entity.

Several viral-based, antineoplastic strategies are being evaluated for treatment of colorectal cancer and liver metastases. Most use replication-defective viruses as vectors to transfer therapeutic genes that encode protein products such as cytokines, prodrugs, and tumor suppressors (10–13). Another promising strategy involves the use of replication-competent viruses and exploits their natural ability to infect and lyse tumor cells (14). These oncolytic viruses are genetically engineered to be less virulent to normal tissues and more specific toward tumor cells. G207 is a second-generation, multi-mutated, replication-

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competent herpes simplex virus type-1 (HSV-1),[2] which has demonstrated impressive oncolytic activity in several neurological malignancies, while sparing normal neural tissue (15, 16).

This engineered virus, based on the wild-type HSV-1 (F-strain), contains deletions of both copies of the $\gamma_1 34.5$ gene, which results in attenuated neurovirulence (17, 18). Interruption of the $U_L 39$ gene restricts replication of the G207 virus to rapidly dividing cells (19). $U_L 39$ codes for ICP6, the large subunit of ribonucleotide reductase, which is a key enzyme required for DNA synthesis and HSV replication. Nondividing cells are deficient in this protein, whereas rapidly dividing cancer cells express it freely (20, 21). Viral replication can only take place in the presence of this enzyme; thus, replication of G207 is limited to dividing cells. In addition, G207 was created with the marker gene *lacZ*, which produces a histochemically identifiable protein product. Cells infected with G207 will turn blue when exposed to X-gal solution. Finally, G207 has two built-in safety mechanisms: expression of herpes thymidine kinase, which renders the virus sensitive to ganciclovir therapy; and temperature sensitivity, which halts viral activity in the febrile host (16).

G207 was originally designed for treatment of neurological malignancies, because wild-type HSV is naturally neurotropic (16). Therapeutic safety and efficacy in animal model neurological tumors encouraged us to study the effects of G207 in solid tumors outside the central nervous system. We report the evaluation of G207 for colorectal cancer and liver metastases *in vitro* and in animal models, and demonstrate this virus to be promising for the treatment of this disease.

MATERIALS AND METHODS

G207 virus

G207, a gift from Dr. S. D. Rabkin, was constructed as described previously, with deletions of both copies of the $\gamma_1 34.5$ gene and insertion of the *Escherichia coli lacZ* gene into the $U_L 39$ sequence of the R3616 mutant (16). African green monkey kidney cells (Vero cells, ATCC, Rockville, Md.) were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum under standard cell culture conditions. Virus was propagated on Vero cells at an MOI (multiplicity of infection, number of viral particles/cell) of 0.02 at 34°C, harvested after 2 days, and subjected to freeze-thaw lysis and sonication to release G207 from the cell fraction. Cell lysates were clarified by centrifugation (300 × *g* for 10 min at 4°C) and viral supernatants were stored at −80°C. Viral titers were determined by standard plaque assay on Vero cells.

[2] Abbreviations: HSV-1, herpes simplex virus type-1; i.p., intraperitoneal; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFU, plaque-forming units.

Cell culture

Five human colorectal cancer cell lines were used in this study. Four (C18, C29, C85, and C86) were isolated and characterized at Memorial Sloan-Kettering Cancer Center. The fifth, HCT 8, was obtained from the ATCC. Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified atmosphere and subcultured twice a week.

In vitro assay of infection and lysis of human colorectal cancer cells

Each of the five cell lines was plated onto flat-bottom 96-well microtiter plates (Becton Dickinson, Franklin Lakes, N.J.) at 3×10^4 cells per well. 24 h later, medium was removed and fresh medium with the appropriate concentration of virus was applied in a final volume of 200 μl; MOIs of 0, 0.1, 1.0, and 2.0 were evaluated. All assays were performed in triplicate.

To evaluate infection efficiency among the five cell lines, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fisher Scientific, Fair Lawn, N.J.) staining was performed on identically prepared serial plates at 24, 48, and 72 h postinfection, using a previously described technique (22). The percent of *lacZ*-positive cells was calculated as a measure of infection.

At 24, 72, and 120 h, wells were exposed to 0.25% trypsin; cells were counted on a hemocytometer using trypan blue exclusion to assess the ability of G207 to lyse cells from the five colorectal cancer cell lines.

Determinations of cell doubling time and S-phase fraction

Doubling time

Cells (5×10^4) were plated onto T-25 flasks (Costar Corporation, Cambridge, Mass.) in 10 cc of medium. Trypsinization of the monolayer and counting by hemocytometer was performed at 24 h intervals. Data were plotted and doubling time was calculated.

Cell cycle analysis

Cells in log-phase growth were harvested by trypsinization, fixed in 80% ethanol, and stored at −20°C until analysis. Subsequently, cell suspensions were digested in DNase-free RNase (Boehringer Mannheim, Indianapolis, Ind.) for 20 min at 37°C and then stained with propidium iodide solution (50 μg/ml, Molecular Probes, Eugene, Oreg.). Cell cycle analysis was performed on FACScan equipped with FACStation running CellQuest software (Becton Dickinson, San Jose, Calif.). A forward angle, light scatter threshold trigger was used to eliminate debris. Cell clumps were removed using analysis gates on either fluorescence pulse width or height vs. pulse area (integral). Data were analyzed for 1×10^4 to 2×10^4 cells per sample.

Viral growth curves

To demonstrate replication of virus in susceptible cells, viral growth curves were established in two representative cell lines (HCT 8 and C18) as described previously (23). Cells (5×10^5) were plated in 6-well plates and infected with G207 at an MOI of 0.01 (5×10^3 PFU). Successive well contents were collected by cell scraping at 0, 12, 24, 36, and 48 h postinfection and subjected to three cycles of freeze-thaw lysis, sonication, and centrifugation at 2000 rpm for 10 min at 4 °C.

Supernatants were plated on confluent Vero cells in 6-well plates at varying dilutions and recovered titers were determined through standard plaque assay.

Suppression of flank tumor growth in athymic rats

All animal work was performed under guidelines approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Athymic rats were housed in pathogen-free quarters in the animal facility. Animals were anesthetized with intraperitoneal (i.p.) injections of pentobarbital (50 mg/kg). Two injections of 2×10^6 tumor cells in 50 μl of culture medium were administered to each side of the animal for a total of four tumors per animal, and tumor growth was measured with calipers three times per week. Estimates of tumor volumes were calculated using the formula for a prolate spheroid, $4/3(\pi)ab^2$, with 'a' as the radius of the long axis and 'b' as the radius of the short axis in millimeters. When tumor volume reached ~50 mm^3, 1×10^7 plaque-forming units (PFU) of active G207 or heat-inactivated virus (65°C for 20 min) in 50 μl of culture medium was injected directly into xenografts. Three cell lines (C85, C86, and HCT8) were selected for the flank tumor model. Sixteen tumors were evaluated for each cell line; eight were treated with active G207 and eight with inactivated virus control. Tumor measurements and animal weights were followed regularly. All control animals (n=6) had to be killed at 3 wk due to ulceration of the skin overlying the tumor. Four of the six G207-treated animals were maintained for 8 wk to evaluate duration of response.

Treatment of hepatic metastases with regional vascular infusion of G207

An established model of hepatic micrometastases was chosen to assess the role of regional hepatic administration of G207. Male Buffalo rats (National Cancer Institute, Bethesda, Md.) that receive intrasplenic injections of 1×10^6 syngeneic Morris hepatoma McA-RH7777 cells (ATCC No. CRL 1601) will reliably develop between 60 and 100 countable liver metastases within 3 wk of tumor challenge. Hepatoma cells were maintained in culture and periodically implanted in flanks to ensure tumorigenicity. Infection and lysis of hepatoma cells with G207 was confirmed *in vitro*.

To evaluate the ability of G207 to suppress experimental hepatic metastases, 17 rats underwent laparotomy, splenic tumor challenge, and splenectomy according to the model of Lafreniere and Rosenberg (24). One week later, portal vein infusions were performed with 1×10^8 PFU of G207 (n=7) in 0.5 ml phosphate-buffered saline (PBS) or an equal volume of PBS alone (n=10).

Portal vein infusion technique

Rats were anesthetized with i.p. pentobarbital (50 mg/kg). Midline laparotomy incisions were made and the anterior pyloric branch of the portal vein was exposed. Under an operating microscope, 6–0 silk suture (USSC, Norwalk Conn.) was used to obtain proximal and distal control of the vessel. Through a small venotomy, a polyethylene catheter (internal diameter = 0.28 mm, Becton Dickinson) with a beveled tip was fed into the portal vein and temporarily secured with a microaneurysm clip (Becton Dickinson). By this technique, homogeneous hepatic distribution was confirmed with India ink in a few animals. Experimental animals were infused with 1×10^8 PFU of G207 in 500 μl of PBS or an equal volume of PBS alone. Eleven days after treatment, rats were killed and liver nodules were counted.

Determination of viral persistence and dissemination

Histochemical analysis

Tumors and organs (brain, heart, lung, liver, kidney, spleen, testes, small bowel, and skeletal muscle) were harvested from animals in both *in vivo* experiments at 1, 7, and 14 days post G207 injection. Additional flank tumors were harvested and sectioned at day 21 postinfection. Specimens were frozen in Tissue-Tek embedding medium (Sakura Finetek, Torrance, Calif.) and sectioned by cryotome. Sections were fixed with 1-% glutaraldehyde and evaluated for β-galactosidase expression by staining with X-gal solution. Slides were subsequently counterstained with Nuclear Fast Red (Sigma, St. Louis, Mo.). Tissues harvested from uninfected animals were used as negative controls.

Quantitative PCR analysis

To further define persistence of infection and extent of dissemination, we performed quantitative polymerase chain reaction (PCR) analysis on genomic DNA extracted from liver, lung, brain, kidney, testes, and serum of Buffalo rats at day 1 (n=2) and day 7 (n=2) postportal vein infusion of 1×10^8 PFU of G207. Additional liver and serum samples (n=2) were analyzed at day 14. Serial femoral artery blood samples were collected from a single rat at 0, 5, 10, 30, and 60 min after portal vein infusion of 1×10^8 PFU of G207. Serum samples were extracted with phenol/chloroform and precipitated using yeast tRNA. Genomic DNA extraction was performed on all tissues. Standard curves were established by doping uninfected liver and serum with known quantities of the G207 virus prior to DNA extraction. Real-time quantitative PCR was performed using an ABI Prism 7700 Sequence Detector (PE Biosystems), as described previously (25, 26). Sense (5′-ATGTTTCCCGTCTGGTCCAC-3′), antisense (5′-CCCTGTCGCCTTACGTGAA-3′) primers, and a dual-labeled fluorescent TaqMan probe (5′-6FAM-CCCCGTCTCCATGTC-CAGGATGG-TAMRA-3′) were designed to span the 111-bp fragment of the HSV ICP0 (immediate early gene). Additional sense (5′-CGCCTACCACATCCAAGGAA-3′), antisense (5′-GCTGGAATTACCGCGGCT-3′) primers, and TaqMan probe (5′-JOE-TGCTGGCACCAGCTTGCCCTC-TAMRA-3′) for the 187-bp 18S rRNA coding sequence were used in the same reaction to normalize the amount of total DNA. Samples were subjected to 40 cycles of PCR (stage 1: 50 °C, 2 min; stage 2: 95 °C, 10 min; stage 3: 95 °C, 15 s; 60 °C, 1 min; stage 4: 25 °C, soak). The AmpliTaq Gold nuclease cleaves a fluorescent dye (FAM or JOE) from the nonextendable probe, liberating it from the proximity of an associated quencher (TAMRA). Probe binding is a requisite for primer extension. The sequence detector is coupled to a charge-coupled device camera, which records the fluorescent emission spectra from individual wells at 500–650 nm with each cycle. Specificity for amplified product is conferred by both probe and primer sets, obviating the need for Southern blot analysis of the PCR product.

RESULTS

In vitro assay of infection and lysis of human colorectal cancer cells

In vitro infection efficiency was measured by staining for β-galactosidase with X-gal solution 24, 48, and

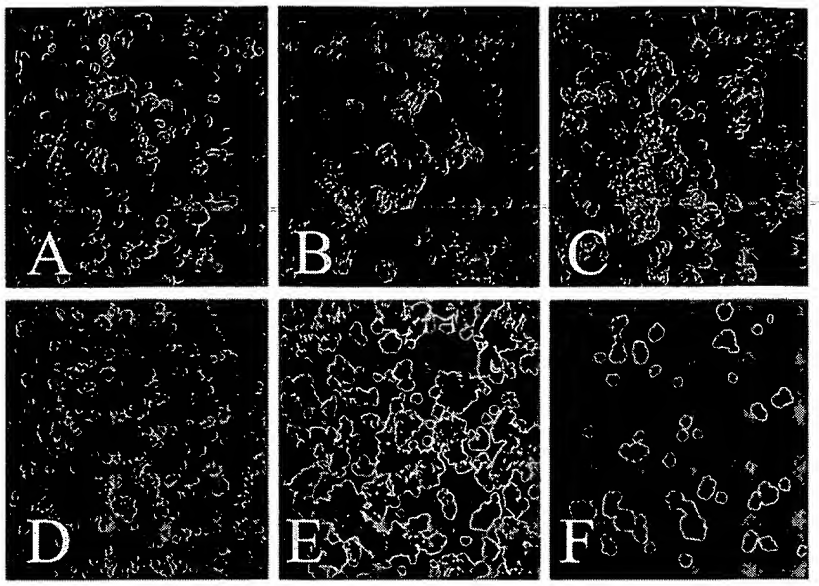

Figure 1. LacZ expression in C85 colorectal cancer cells after infection with G207 over time at MOI 0.1 (original magnification: ×100). *A–C*) Control cells infected with heat-inactivated G207 and stained with X-gal solution at 24, 48, and 72 h, respectively. Cells show no evidence of G207 infection and continue to divide appropriately. *D—F*) Parallel plates infected with active G207. In panel *D* (24 h), only a few cells demonstrate G207 infection (*lacZ* positive), which increases dramatically in panel *E* (48 h). *F*) Most cells have been lysed by G207 and all remaining cells show evidence of infection.

72 h after treatment with G207 at several MOIs (**Fig. 1**). G207 demonstrated impressive infection efficiency in three of the five cell lines tested (C85, C86, and HCT 8) and more moderate infectivity in C18 and C29, as determined by calculating the percent of *lacZ*-positive cells (**Fig. 2***A*). Infection correlated with cell kill. The highly susceptible cell lines (C85, C86, and HCT8) demonstrated 76% or better cell kill at an MOI of 0.1, 72 h after infection, whereas the moderate responders (C18 and C29) showed 31% or less cell kill under the same conditions (Fig. 2*B*). All colorectal cancer cell lines tested demonstrated appreciable susceptibility to G207 cytotoxicity as compared with controls, even at the lowest MOI (0.1). In all cell lines tested, infection efficiency correlated with percent tumor cell lysis.

Determinations of cell doubling time and S-phase fraction

In vitro doubling time and S-phase fraction were determined for each colorectal cancer cell line. Both parameters were determined from cell populations in log-phase growth. The two cell lines that displayed moderate susceptibility to G207 (C18 and C29) had doubling times of 44 and 48 h and S-phase fractions of 9.2% and 8.6%. By comparison, the three cell lines that showed strong susceptibility to the virus (C85, C86 and HCT 8) had shorter doubling times (19–26 h) and greater S-phase fractions (20.1%–36.6%, **Table 1**). These results demonstrate a direct correlation between the proliferative indices of the cancer cell lines and their susceptibility to G207 therapy *in vitro* ($R = +0.8$, linear regression analysis of infection percentage at MOI 1.0 vs. S-phase fraction).

Viral growth curves

In vitro viral growth curves were performed to demonstrate the ability of G207 to replicate in human colorectal cancer cells. For this experiment, two cell lines were evaluated: a strong responder to *in vitro* cytotoxicity (HCT 8), with a short doubling time (19 h) and high S-phase fraction (37%), and a less responsive line (C18) with a longer doubling time (44 h) and lower S-phase fraction (9%). The results are depicted in **Fig. 3**. At the 0 time point, ~50% of viral particles are recovered from the initial infection in both cell lines. By 12 h, there is a modest drop of recovered viral titers by an additional 50%, coinciding with viral disassembly within the infected cell. Subsequently, in the responsive line (HCT 8), the titers begin to rise at 24 h and by 48 h are increased by 1.5 logs over the number of viral particles initially added to the well. Conversely, in the less responsive line (C18), there is no rise in recovered titers of G207, suggesting that this slowly dividing cell line does not foster efficient viral replication.

Suppression of flank tumor growth in athymic rats

Direct injection of G207 suppressed xenograft tumor growth significantly in all three cell lines tested as compared with controls (**Fig. 4***A, B*). HCT 8 xenografts were most susceptible, with responses in 8/8 treated tumors (4/8 partial responses and 4/8 complete responses). C86 xenografts demonstrated similar results (4/8 partial responses and 2/8 complete responses). C85 tumors were not completely suppressed by G207 injection, but growth rate was reduced significantly as compared with control.

As a group, G207-treated animals maintained their health as well or better than control animals.

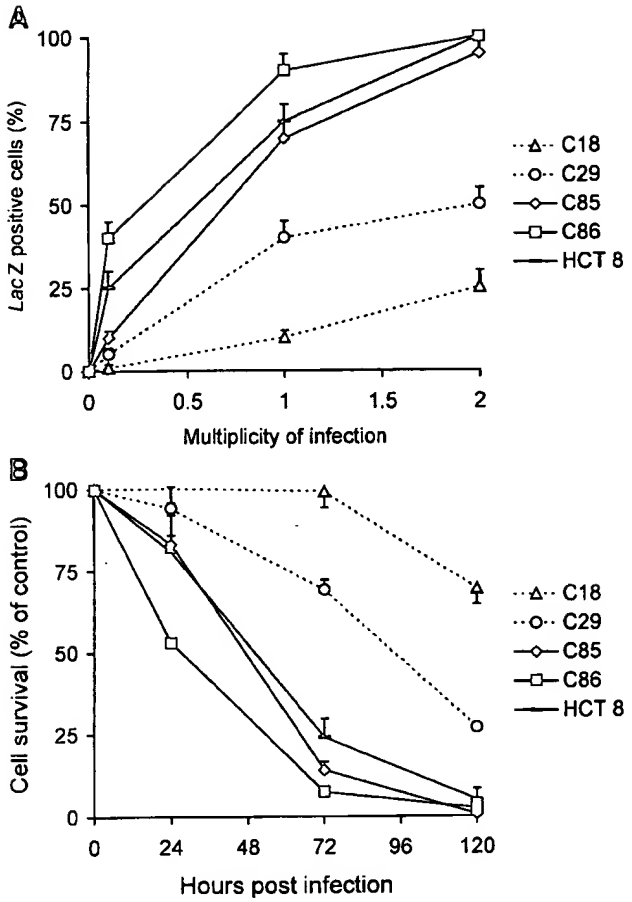

Figure 2. *In vitro* effects of G207 infection on five human colorectal cancer cell lines. *A)* Summary data for all five colorectal cancer cell lines relating percent of *lacZ*-positive cells to MOI. *B)* Cell survival, represented as percent of control, for the five colorectal cancer cell lines at various times after exposure to G207 at an MOI of 0.1.

G207-treated animal gained significantly more weight than controls during the 20 day experiment (100 ± 16 g vs. 44 ± 9 g, $P<0.5$). All animals that developed ulceration in the skin overlying their tumors had to be killed. This occurred in 6/6 control animals and in 2/6 of the G207-treated animals by day 20 posttumor inoculation. The four

TABLE 1. *Correlation of cell line proliferative indices and G207 infection and cytotoxicity*

Cell line	Doubling time (hours)	S-phase fraction (percent)[a]	% blue cells MOI 1.0 (24 h)[b]	% cell kill MOI 0.1 (72 h)[c]
C18	44	9.2, (CV = 5.7)	10 ± 2	1 ± 5
C29	48	8.6, (CV = 5.3)	40 ± 5	31 ± 3
C85	26	20.1, (CV = 4.8)	70 ± 10	86 ± 3
C86	24	26.5, (CV = 5.1)	90 ± 5	93 ± 1
HCT 8	19	36.6, (CV = 4.9)	75 ± 5	76 ± 6

[a] CV represents coefficient of variance for FACScan analysis. [b] From data presented in Fig. 2*A*. [c] From data presented in Fig. 2*B*.

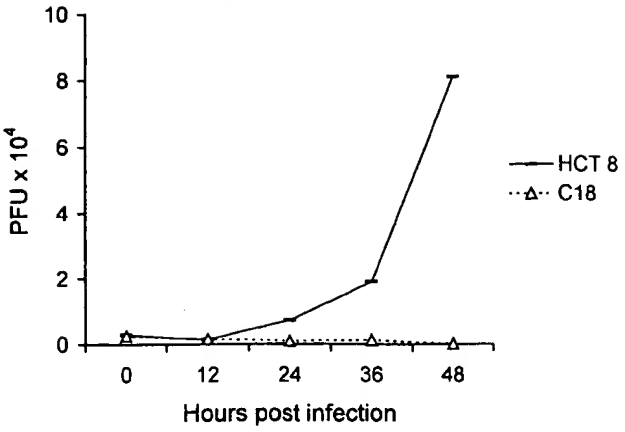

Figure 3. Viral growth curves for G207 in two representative colorectal cancer cell lines. Note that the amount of virus recovered from the HCT 8 wells (cell line doubling time of 19 h) increases 1.5 logs over 48 h. Conversely, the amount of virus recovered from the C18 wells (cell line doubling time of 44 h) falls well below the 5×10^3 PFU that was initially plated.

G207-treated animals that showed no evidence of ulceration were evaluated for duration of response for an additional 8 wk. Tumors in these animals did not continue to grow during this period, and the animals continued to groom and gain weight appropriately.

Treatment of hepatic metastases with regional infusion of G207

Initial studies confirmed that the Morris hepatoma McA-RH7777 cell line is sensitive to G207 *in vitro* (data not shown). In the efficacy study, animals that received portal infusions of G207 7 days after splenic tumor challenge recovered well, grooming and gaining weight equal to the PBS-treated controls. At death (day 11 postinfusion), G207-treated livers contained far fewer nodules than PBS-treated controls (13 ± 10 nodules vs. 80 ± 30 nodules, $P<0.05$, **Fig. 5**).

Determination of viral persistence and dissemination

Histochemical analysis

Cryosections prepared from athymic rat organs (brain, heart, lung, liver, kidney, spleen, testes, small bowel, and skeletal muscle) harvested at 1, 7, and 14 days post G207 flank tumor injection demonstrated absence of β-galactosidase expression. Conversely, strong expression was found in all tumors that received 1×10^7 PFU via direct, single intratumoral injection. Tumors that demonstrated only partial response to therapy were evaluated at 21 days and found to have persistence of *lacZ* positivity in a

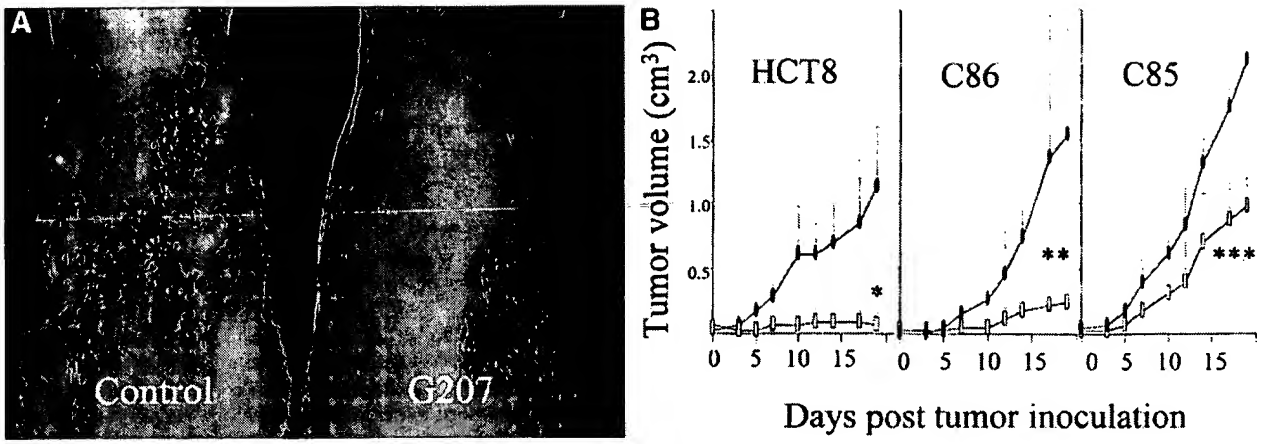

Figure 4. Results of *in vivo* administration of G207. *A)* Representative nude rats demonstrating effects of injection with heat-inactivated virus (control) or with viable G207. Animals are shown 20 days after a single injection (1×10^7 PFU) of each tumor. *B)* Summary of *in vivo* injection experiments for three different colorectal cancer cell lines. Tumor volume is illustrated at various times after a single injection with heat-inactivated virus (closed circles) or active G207 (open boxes, $*P<0.0001$; $**P<0.01$; $***P<0.05$, *t* test).

depot-like distribution. There was less overall necrosis witnessed in these specimens, suggesting less infectivity of the virus in neighboring cells.

In the portal vein infusion model, all organs (with the exception of liver) were negative for β-galactosidase activity. Nontumor liver parenchyma demonstrated scant positive expression overall (less than 1 blue cell/LPF). This was found in half the sections studied (18/36), with the remainder showing no blue cells in the normal parenchyma. In animals with established gross hepatic tumor, which were then exposed to portal vein G207, X-gal staining was impressive. Twenty-four and 48 h after exposure to G207, ~two-thirds of existing liver nodules (~50/80) had strong expression of β-galactosidase in grouped arrays (**Fig. 6**). About half of the nodules had a single focus, whereas the remainder had multiple positive regions with up to five per nodule. Peripherally located tumor-associated plaques abutted but did not cross the border into the adjacent normal liver parenchyma.

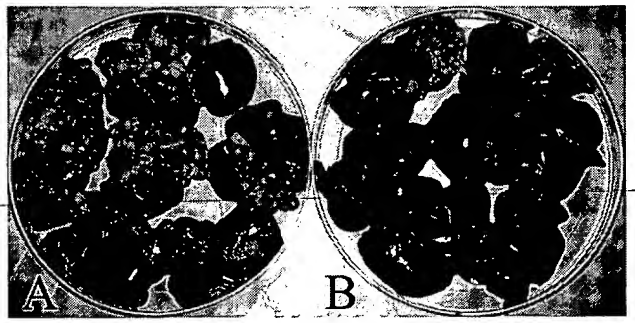

Figure 5. Effects of hepatic portal vein infusion of G207. Representative livers 11 days after portal vein infusion with PBS (*A*) or G207 (*B*). Portal vein infusions were performed 7 days after splenic injection of tumor cells.

Quantitative PCR analysis

Quantitative PCR analysis of tissue and serum samples was performed to further define the presence, persistence, and extent of dissemination of G207 in animals that were infected via portal vein infusion. By establishing a standard curve with 1, 5, 10, 10^3, and 10^5 PFU, we first determined our limit of reliable detection to be 10 PFU per 50 mg of tissue. Upon analysis of the various organs (liver, lung, kidney, brain, and testes), only liver clearly demon-

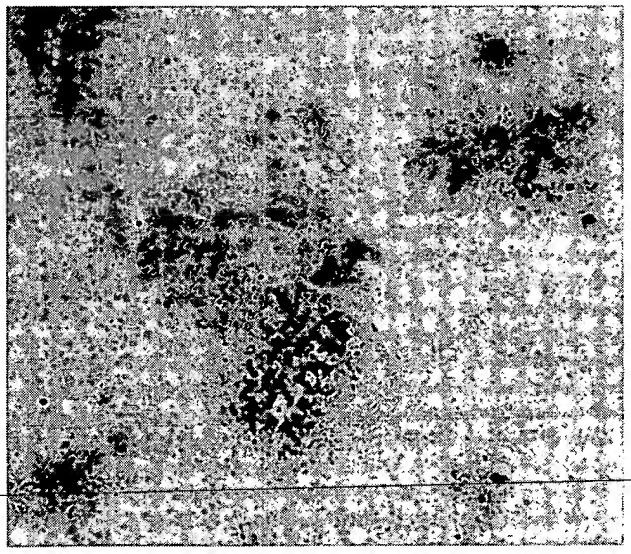

Figure 6. Representative frozen section of hepatic tumor nodule (original magnification: ×100) from animal with established liver metastases 48 h after portal vein infusion of 1×10^8 PFU of G207. Section is stained with X-gal solution and counterstained with Nuclear Fast Red. Multiple blue plaques are evident, demonstrating impressive distribution of G207 infection within a single nodule.

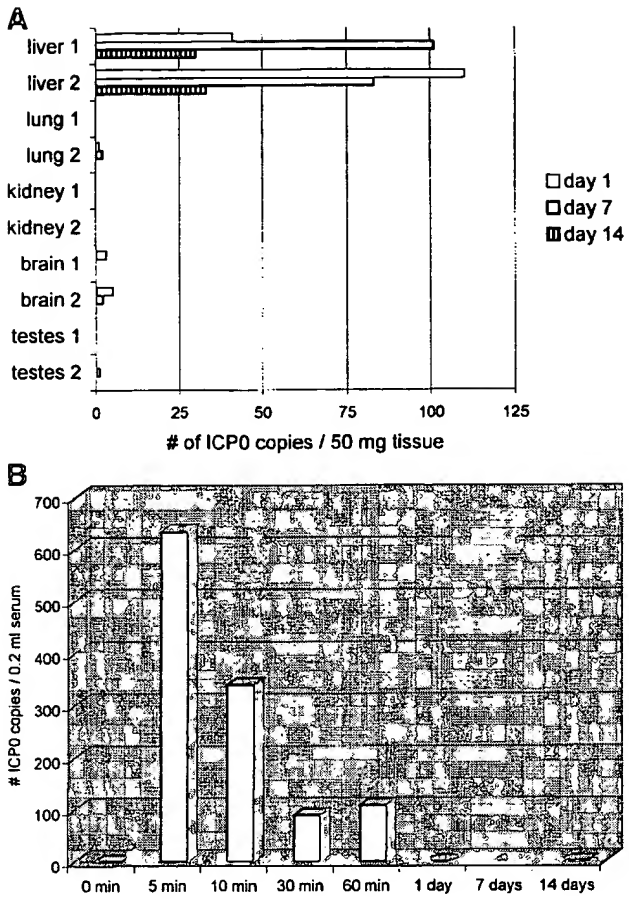

Figure 7. Summary data for quantitative PCR analysis using primers and probe for the HSV-ICP0 DNA sequence. ICP0 copy number correlates with plaque-forming units used in standard curve. *A)* Number of ICP0 copies per 50 mg of tissue at days 1, 7, and 14 after portal vein infusion of 1×10^8 PFU of G207 in Buffalo rats (only liver tissue was analyzed at day 14). *B)* Number of ICP0 copies per 0.2 ml of serum isolated from the femoral artery of Buffalo rats at 0, 5, 10, 30, and 60 min and 1, 7, and 14 days after portal vein infusion with G207.

strated the presence of the HSV-ICP0 DNA sequence at any time point evaluated (**Fig. 7***A*). Minimal signal was observed in lung, brain, and testes, but based on our standard curve, this expression was below the limit of reliable detection. Furthermore, livers exposed to portal venous G207 demonstrated persistence of the viral DNA sequence at 14 days. Analysis of serum samples showed significant recovery of viral DNA in the peripheral arterial blood at 5 min (> 600 copies/0.2 ml) postportal venous infusion, which decreased over the first hour and was absent from 24 h and beyond (Fig. 7*B*).

DISCUSSION

Our study evaluates the efficacy of the multi-mutated, attenuated HSV-1, replication-restricted virus G207 to treat a common nonneurological neoplasm. We have shown that this agent can infect, replicate within, and kill human colon cancer cells, and that cell line characteristics such as doubling time and S-phase fraction may play a role in determining sensitivity to G207. We have also demonstrated two effective methods of therapeutic delivery in direct intratumoral injection and regional vascular infusion, and have investigated the extent of dissemination and persistence of viral protein and DNA through assay of marker gene product and quantitative PCR.

Some of the earliest work with replicating recombinant HSV vectors was directed at development of antiherpetic vaccines, with the goal of establishing a lasting immune response in infected hosts (27, 28). As better understanding of the factors responsible for HSV virulence (18) (17) and replication (19, 29) developed, it became clear that these mutants could be used to treat malignancies. Neurological tumors were first targeted due to the natural neurotropism of herpes viruses. Selective infection and destruction of tumors within the central nervous system, with sparing of normal neurons, has been demonstrated by several investigators (15, 16, 30, 31).

More recently, several groups have tested various recombinant, replicating HSV mutants to treat nonneurological malignancies with success (20, 23, 32). Of note is work by Kucharczuk et al. (23), who demonstrated safety and efficacy of HSV-1716 (a γ34.5 deleted mutant) in the treatment of experimental human malignant mesothelioma cell lines *in vitro* and then in an animal model of peritoneal disease. PCR analysis was used to demonstrate that virus was detectable in tumor, but not in normal tissues.

G207 is a second-generation, multi-mutated HSV-1 that was developed by taking the double γ34.5-deleted F-strain mutant R3616 and inactivating the DNA sequence coding for viral ribonucleotide reductase. This was done through an insertional mutation with the *E. coli lacZ* gene (15, 16). Thus, not only is this virus attenuated in virulence, but its replication is restricted to cells that presumably produce higher levels of endogenous ribonucleotide reductase (i.e., rapidly dividing malignant cells) and can be detected by assaying for production of β-galactosidase. Another benefit derived from using multiple mutations to create G207 is the reduced likelihood of reversion to wild-type disease-causing parent virus.

The precise mechanism for viral cell kill is still being deciphered. In susceptible cell lines, characteristics of normal HSV infection such as ballooning and formation of multinucleated giant cells are observed. This observation and evidence of viral replication, as demonstrated through viral growth

curves, support active, productive infection with completion of the viral lytic life cycle as one mechanism contributing to the death of infected cells. Moreover, several authors have supported the notion that deletion of γ34.5 may promote apoptosis in susceptible cells (17, 18, 33). The carboxyl terminus codes for a protein product that bears homology to a highly conserved mammalian protein known as GADD 34. This protein is up-regulated when cells are placed under stressful conditions (e.g., serum starvation or viral infection) and acts to preclude protein synthesis. In the absence of this protein, infected susceptible cells sense the insult and respond by entering the pathway to programmed cell death instead of reevaluating and attempting to repair and persevere.

As for restricted replication, it is known that at least 45 of the 84 characterized HSV genes can be inactivated or deleted and the virus will still replicate in cultured cells, with some altered characteristics (34). Inactivation of the gene encoding ICP6 (or viral ribonucleotide reductase) is well described. This enzyme plays a key role in DNA synthesis in prokaryotes and eukaryotes. Evidence exists to suggest that an ICP6-inactivated mutant herpes virus may be able to compensate for its deficit by using cellular ribonucleotide reductase (19, 29, 35, 36). Thus, rapidly dividing cells, which presumably express higher levels of ribonucleotide reductase, may serve as more suitable hosts for G207 replication. Our laboratory is currently working on defining this relationship in our models.

Our data indicate that G207 efficiently infects experimental human colorectal cancer cells. All five cell lines tested responded to treatment *in vitro*. Three of the cell lines (C85, C86, and HCT 8) had remarkable responses by 3 and 5 days postinfection, even at a low MOI of 0.1. These strong responders exhibited more rapid cell division as demonstrated by measurements of cell doubling time and S-phase fraction. The relationship between cellular proliferative rate and responsiveness to G207 was observed in the viral growth curves. G207 demonstrated active replication within the rapidly dividing HCT 8 cell line, whereas the cell line with a slower turnover (C18) did not support successful viral growth. Furthermore, HCT 8 cells had the highest S-phase fraction *in vitro* (36.6%) and the greatest *in vivo* tumor growth inhibition of the three cell lines tested in the direct intratumoral injection model, with 4/8 tumors sustaining complete responses. The observed correlation between cell line proliferation and susceptibility to G207 *in vitro* may serve as a clinically relevant predictor of *in vivo* therapeutic response.

In addition to examining direct intratumoral injections, we investigated the efficacy of G207 as a possible agent for regional antineoplastic therapy. Introducing the virus by selective intravascular infusion is appealing since it allows a diffuse distribution of virus within the tumor. The same hypervascularity that the tumor creates to allow its own continued growth could be exploited to increase viral delivery. HSV-1 is a large DNA virus that approaches 200 nm in diameter, raising a concern that infectious particles would not cross the endothelium and enter tumor cells. We examined this issue using a well-established syngeneic model of hepatic metastases, which involves seeding the liver with tumor via portal inoculation, a path identical to that taken by colorectal liver metastases in human beings (3). Our results suggest that regional infusion with G207 may be valuable for treatment of unresectable liver malignancies or may be useful as an adjuvant to surgical resection to reduce postoperative local recurrence.

Although G207 is an attenuated virus with restricted replication, therapeutic use of replicating viruses still raises concerns of dissemination. In our study, we used two methods of detection. The first, X-gal staining, requires that the virus infects the cell, transfers its genetic material, and the transferred marker gene functions to produce a protein (β-gal). We evaluated various tissues (including tumor) for the presence of the marker gene product. In the flank tumor model, only tumor demonstrated positive results. In the portal vein infusion model, high expression was demonstrated in established tumor with infrequent expression in normal hepatic parenchyma. All other organs were negative for G207 infection by this detection method.

The second method, real-time quantitative PCR, detects presence of the herpes immediate-early gene ICP0, which plays a role in cell cycle regulation in infected cells (34). Unlike X-gal staining, this sensitive technique does not rely on the expression of viral genes to identify infected cells. We analyzed liver tissue as our target organ, lung tissue because it receives most of the hepatic venous blood flow, brain because of natural herpes tropism, kidney because of its function of filtration, and testes because this is an area with inherent rapid cell division. We found presence of the HSV-ICP0 sequence in liver of animals that received 1×10^8 PFU of portal G207 up to 14 days and in the serum of an animal soon after infusion of virus, but not in any other organs examined. Moreover, no mortalities were observed, and G207-treated rats continued to groom and gain weight appropriately at therapeutic doses.

Primates serve as the natural host for HSV-1, which may explain selective infection of human tumor xenografts in rodents. Our hepatic metastases model addresses this issue of species tropism. Morris hepatoma McA-RH7777 is a syngeneic tumor cell line developed and passaged in Buffalo rats. In this model, G207 demonstrates impressive tumor cell

selectivity. These results suggest that G207 therapy is both tumor specific and effective at suppressing tumor growth in this model.

The future of oncolytic viral therapy is evolving. Recent advances in the literature report methods to improve potency of attenuated herpes viruses against malignant cells. Such advances include adding ionizing radiation as an adjuvant to enhance the virus's ability to replicate (37) and using attenuated, replicating viruses as helper viruses for packaging immunostimulatory cytokine genes (38). Another area worthy of investigation is cotreatment with antiviral agents. G207 expresses HSV-thymidine kinase and is reported to be hypersensitive to ganciclovir (16). Future experimentation should investigate the added efficacy of combining the natural oncolytic properties of G207 with this suicide gene therapeutic strategy, bearing in mind that treatment with antiviral agents could, in theory, inactivate the virus prematurely.

G207 kills experimental human colorectal cancers efficiently and *in vitro* assays may predict this agent's efficacy *in vivo*. This antineoplastic agent can be administered safely in experimental animals by regional infusion and direct injection and can suppress nonneural tumor growth. These results encourage pursuing future clinical studies of G207 in the treatment of human metastatic colorectal cancers. FJ

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